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Investigation of a new mechanism of desiccation-stress tolerance in *Salmonella*

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Abstract.

Low-moisture foods (LMF) are increasingly involved in foodborne illness. While bacteria cannot grow in LMF due to the low water content, pathogens such as *Salmonella* can still survive in dry foods and pose health risks to consumer. We recently found that *Salmonella* secretes a proteinaceous compound during desiccation, which we identified as OsmY, an osmotic stress response protein of 177 amino acids. To elucidate the role of OsmY in conferring tolerance against desiccation and other stresses in *Salmonella enterica* serovar Typhimurium (STm), our specific objectives were: (1) Characterize the involvement of OsmY in desiccation tolerance; (2) Perform structure-function analysis of OsmY; (3) Study OsmY expression under various growth- and environmental conditions of relevance to agriculture; (4) Examine the involvement of OsmY in response to other stresses of relevance to agriculture; and (5) Elucidate regulatory pathways involved in controlling osmY expression. We demonstrated that an osmY-mutant strain is impaired in both desiccation tolerance (DT) and in long-term persistence during cold storage (LTP). Genetic complementation and addition of a recombinant OsmY (rOsmY) restored the mutant survival back to that of the wild type (wt). To analyze the function of specific domains we have generated a recombinant OsmY (rOsmY) protein. A dose-response DT study showed that rOsmY has the highest protection at a concentration of 0.5 nM. This effect was protein-specific as a comparable amount of bovine serum albumin, an unrelated protein, had a three-time lower protection level. Further characterization of OsmY revealed that the protein has a surfactant activity and is involved in swarming motility. OsmY was shown to facilitate biofilm formation during dehydration but not during bacterial growth under optimal growth conditions. This finding suggests that expression and secretion of OsmY under stress conditions was potentially associated with facilitating biofilm production.

OsmY contains two conserved BON domains. To better understand the role of the BON sites in OsmY-mediated dehydration tolerance, we have generated two additional rOsmY constructs, lacking either BON1 or BON2 sites. BON1-minus (but not BON2) protein has decreased dehydration tolerance compared to intact rOsmY, suggesting that BON1 is required for maximal OsmY-mediated activity. Addition of BON1-peptide at concentration below 0.4 μM did not affect STm survival. Interestingly, a toxic effect of BON1 peptide was observed in concentration as low as 0.4 μM. Higher concentrations resulted in complete abrogation of the rOsmY effect, supporting the notion that BON-mediated interaction is essential for rOsmY activity.

We performed extensive analysis of RNA expression of STm undergoing desiccation after exponential and stationary growth, identifying all categories of genes that are differentially expressed during this process. We also performed massively in-parallel screening of all genes in which mutation caused changes in fitness during drying, identifying over 400 such genes, which are now undergoing confirmation. As expected OsmY is one of these genes.

In conclusion, this is the first study to identify that OsmY protein secreted during dehydration contributes to desiccation tolerance in *Salmonella* by facilitating dehydration-mediated biofilm formation. Expression of OsmY also enhances swarming motility, apparently through its surfactant activity. The BON1 domain is required for full OsmY activity, demonstrating a potential intervention to reduce pathogen survival in food processing. Expression and fitness screens have begun to elucidate the processes of desiccation, with the potential to uncover additional specific targets for efforts to mitigate pathogen survival in desiccation.

Summary Sheet

Publication Summary

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Training Summary

Trainee Type	Last Name	First Name	Institution	Country
Ph.D. Student	Karasik	Lyliya	ARO Volcani	Israel
Postdoctoral Fellow	Desai	Prerak	UCI	USA
M.Sc. Student	Bharill	Varun	UCI	USA
M.Sc. Student	Saraogi	Parag	UCI	USA

Contribution of Collaboration

The two teams engage in frequent email communication regarding the results and potential directions. The Israeli team has a long expertise in Salmonella desiccation and the US team specializes in transcriptomics, mutant construction, and mutant screens. Thus, the collaboration resulted in a synergistic outcome. The Israeli team performed the desiccation experiments and purified the total mRNA, which was sent to the US team, where it was further purified and subjected to RNA-sequencing and bioinformatics analysis. The US team constructed specific isogenic mutants which were used by both teams to test the dehydration tolerance of these mutants. The US team performed transposon screens. The Israeli team constructed over-expressing mutants and proteins, and performed the toxicity and biofilm experiments. Altogether, the collaboration has enabled an efficient and a better study which should hopefully be published in a high tier journal.

Achievements. Significance of main scientific achievements or innovations.

Reduction of moisture is a common approach to limit bacterial multiplication in food. However, recent outbreaks of salmonellosis linked to consumption of low-moisture foods (LMF) have raised interest in understanding how non-typhoidal *Salmonella enterica* persist during desiccation. We recently found that during dehydration, high-density *S. enterica* serovar Typhimurium (STm), secretes a compound that facilitates desiccation tolerance of low-density STm and increased bacterial persistence during dehydration and long-term desiccation in cold storage. In the present study we have extended and confirmed the observation that OsmY, an osmotic stress response protein, is an important component of this activity. We further dissected the mode of action of *osmY* and studied the transcriptional profile of dehydrating cells.

We have confirmed the involvement of the OsmY protein in desiccation tolerance (DT) and long-term persistence (LTP). An osmY- knockout mutant did not produce a protective secretion and was more susceptible to 22 h dehydration and to long-term persistence (LPT; 4 weeks, 4^{o}C) during cold-storage, compared to the wild-type (WT) strain. Genetic complementation restored the WT phenotype, while an osmY- mutant transformed with the vector alone (control) retained the mutant's phenotype, supporting a role for OsmY in desiccation tolerance. The protein was further shown to accumulate both intracellularly and extracellularly during STm dehydration. OsmY was detected in the dehydrating bacterial filtrate as early as 8 h with a relative abundance of ca. 4% and its abundance increased to 11% of the total extracellular proteins at 22 h.

The mature OsmY protein consists of 177 amino acids and contains two conserved Bacterial OsmY and Nodulation (BON) sites, each spanning 63 amino acids. A recombinant OsmY (rOsmY) protein was shown to facilitate dehydration tolerance and LTP. A recombinant protein lacking BON1, but not BON2 seems to be less effective than the intact protein, suggesting that BON1 is required for full OsmY activity. Indeed, synthetic BON1 peptide inhibits rOsmY DT activity at comparable molar concentration; yet, higher concentrations is toxic to STm, regardless of the presence or absence of rOsmY.

OsmY has characteristics of a biosurfactant and the mutant has lower swarming motility compared to the wt strain. Confocal microscopy studies utilizing the *osmY* knockout strain and genetic complementation revealed that OsmY protein facilitates biofilm formation during dehydration. Biofilm formation is a well-known bacterial survival strategy against multiple environmental stresses, including desiccation, and *Salmonella* biofilms were previously reported

to display a higher resistance against dehydration than planktonic cells. Therefore, it may be hypothesized that during dehydration, *Salmonella* senses the increased osmotic stress and responds by expressing OsmY which may facilitate biofilm formation in order to protect the cells against the water-less environment.

We analyzed transcriptomes for S. enterica under 915 environmental and growth conditions, the majority of which were experiments from our lab. We correlated expression of each gene with the expression observed for the *osmY* gene. We then used knowledge of functional groups of genes to determine whether any of about 250 known groups of functionally related genes were significantly correlated or anti-correlated with *osmY* gene expression. The top few gene sets are presented in **Table 1** (Appendix). For example, trehalose metabolism is important in dehydration. However, the strongest positive correlation was expression of the *osmY* gene with genes that are negatively-regulated by the QseB/QseC two-component system we had previously defined, of which *osmY* is a member (first line in Table 1). We then set out to determine what systems were regulated at the transcriptional level, and added a goal to determine, which individual genes other than *osmY* were playing a role in desiccation.

We performed RNA profiling of desiccated samples, comparing wt, mutant, and mutant supplemented with exogenous rOsmY in a total of 24 RNAseq libraries from log phase and stationary phase cells. Bacteria were taken for RNA extraction at the following time points 0, 5, 7, 9 and 12 h, corresponding to 0, 50, 70, 90, and 100% water-loss. We observed systematic changes in gene expression for all genes with desiccation (Appendix Fig. 1, Table 2a,b; manuscript in preparation). Enrichment analysis was performed to find classes of genes particularly targeted during desiccation (Appendix Tables 3 and 4). Analysis is ongoing.

While we saw an increase in the relative amount of *osmY* RNA, during dehydration in the wt strain, which also is also experienced similarly by hundreds of other genes, we observed no *osmY* or rOsmY-dependent expression of other genes. The lack of effect of the *osmY* mutant and exogenous rOsmY on these huge changes could mean that OsmY has a structural role that is required to mechanically stabilize the cells, which would otherwise die faster. We sample only RNA expression of these intact surviving cells. The fact that OsmY protein can act exogenously, is consistent with the possibility of a mechanical role.

To confirm that the intact *osmY* gene is indeed under selection during desiccation, we performed an experiment using a library of 200,000 random Tn5 insertion mutants subjected to

repeated wetting and drying for four cycles on two surfaces; plastic, and stainless steel. The former surface is used in food packages, and the latter is a surface extensively used in food processing and preparation. About 470 mutants were under negative selection, crucially, including *osmY*. Also, noteworthy we have used this Tn5 library in hundreds of other selective conditions, none of them being a stress similar to desiccation, and we have never seen statistically significant selection against *osmY* in any of these experiments, indicating that there is a narrow range of conditions where *osmY* is under strong selection. These observations by an entirely independent method, affirm the special relevance of this gene in desiccation. Combined with the transcriptome experiments, this observation implies that *osmY* has a structural and not regulatory role in survival during desiccation, as is fitting, given that it can work exogenously on survival.

We have begun to confirm the relevance of the individual genes and operons that we have identified as being under negative selection, along with *osmY*, in repeated drying and wetting conditions, and to determine the relationship of these genes, if any, to *osmY*. Screening is being done on a 1:1 mixture of a specific knockout deletion of each mutant and the wild type, each expressing a different resistance marker. The ratio of the two gene markers can then be measured by serial dilution on to antibiotic agar plates. Three of the first five deletion mutants screened have a confirmed phenotype.

Agricultural and/or economic impacts of the research findings, if known. OsmY-mediated desiccation tolerance via biofilm formation is a novel strategy, which opens up a new area of research with potential applications in the food safety arena. Since, OsmY was reported to be induced under a variety of stresses; it is possible that OsmY mediates tolerance to other stress we have not yet examined, via a similar mechanism. This notion should be further tested in *Salmonella*, and in other pathogens.

The finding that the BON1-peptide is toxic to the cells might hold promise to use it (or its derivatives) as a novel mean to inactivate *Salmonella* and perhaps other foodborne pathogens. These will require further studies to test the effect of the peptide on various *Salmonella* serovars and on other human pathogen. Our work on other genes that are also of importance in desiccation will provide a wealth of other potential highly focused targets for reducing the risk of *Salmonella* in agriculture.

Changes to original research Plan

The following were the original objectives: (1) Characterize the involvement of OsmY in desiccation tolerance; (2) Perform structure-function analysis of OsmY; (3) Study OsmY expression under various growth- and environmental conditions of relevance to agriculture; (4) Examine the involvement of OsmY in response to other stresses of relevance to agriculture; and (5) Elucidate regulatory pathways involved in controlling osmY expression. Characterizing the involvement of OsmY in desiccation tolerance (Obj. 1) and assessing structure-function relationships of OsmY (Obj. 2) and elucidating regulatory pathways involved in controlling osmY expression (Obj. 5) provided many new exciting avenues that were expanded upon and exploited by the teams, as outlined in the achievements. Consequently, there was time and resources available for only some parts of objective 3 and 4. Some of the proposals in these objectives are now even more relevant in the context of the data we obtained in this funding period. We will request continuation funding that includes those portions of these objectives. For example, we have constructed an *osmY*-promoter fusion to a promoterless GFP and have performed preliminary studies to monitor *osmY* expression through GFP, which will greatly accelerate understanding the expression and compartment of this protein in a wide variety of conditions of relevance to agriculture and food industry.

Publications for Project IS-4671-13CR

Status	Type	Authors	Title	Journal	Vol:pg Year	Coun
Published	Reviewed	*1. Deng, X., Desai, P.T., den Bakker, H.C., Mikoleit, M., Tolar, B., Trees, E., Hendriksen, R.S., Frye, J.G., Porwollik, S., Weimer, B.C., Wiedmann, M., Weinstock, G.M., Fields, P.I. and McClelland, M.*	Genomic epidemiology of Salmonella enterica serotype Enteritidis based on population structure of prevalent lineages.	***Emerg Infect Dis***	20 : 1481-1489 2014	US only
Published	Reviewed	*2. Marzel, A., Desai, P.T., Nissan, I., Schorr, Y.I., Suez, J., Valinsky, L., Reisfeld, A., Agmon, V., Guard, J., McClelland, M., Rahav, G. and Gal-Mor, O.*	Integrative analysis of Salmonellosis in Israel reveals association of Salmonella enterica Serovar 9,12:l,v:- with extraintestinal infections, dissemination of endemic S. enterica Serovar Typhimurium DT104 biotypes, and severe underreporting of outbreaks.	***J Clin Microbiol***	52 : 2078-2088 2014	US only
Published	Reviewed	*3. Pontel, L.B., Scampoli, N.L., Porwollik, S., Checa, S.K., McClelland, M. and Soncini, F.C.*	Identification of a Salmonella ancillary copper detoxification mechanism by a comparative analysis of the genome-wide transcriptional response to copper and zinc excess.	***Microbiology***	160 : 1659-1669 2014	US only
Published	Reviewed	*4. Porwollik, S., Santiviago, C.A., Cheng, P., Long, F., Desai, P., Fredlund, J., Srikumar, S., Silva, C.A., Chu, W., Chen, X., Canals, R., Reynolds, M.M., Bogomolnaya, L., Shields, C., Cui, P., Guo, J., Zheng, Y., Endicott-Yazdani, T., Yang, H.J., Maple, A., Ragoza, Y., Blondel, C.J., Valenzuela, C., Andrews-Polymenis, H. and McClelland, M.*	Defined single-gene and multi-gene deletion mutant collections in Salmonella enterica sv Typhimurium	***PLoS One***	9 : e99820 2014	US only
Published	Reviewed	*5. Henard, C.A., Tapscott, T., Crawford, M.A., Husain, M., Doulias, P.T., Porwollik, S., Liu, L., McClelland, M., Ischiropoulos, H. and Vazquez-Torres, A.*	The 4-cysteine zinc-finger motif of the RNA polymerase regulator DksA serves as a thiol switch for sensing oxidative and nitrosative stress.	***Mol Microbiol***	91 : 790-804 2014	US only
Published	Reviewed	*6. Cheng, Y.,*	rpoS-Regulated core genes	***Appl Environ***	81 : 502-	US only

		Pedroso, A.A., Porwollik, S., McClelland, M., Lee, M.D., Kwan, T., Zamperini, K., Soni, V., Sellers, H.S., Russell, S.M. and Maurer, J.J.	involved in the competitive fitness of Salmonella enterica Serovar Kentucky in the intestines of chickens.	***Microbiol***	514 2015	
Published	Reviewed	*7. Elfenbein, J.R., Knodler, L.A., Nakayasu, E.S., Ansong, C., Brewer, H.M., Bogomolnaya, L., Adams, L.G., McClelland, M., Adkins, J.N. and Andrews-Polymenis, H.L.*	Multicopy Single-Stranded DNA Directs Intestinal Colonization of Enteric Pathogens.	***PLoS Genet***	11 : e1005472 2015	US only
Published	Reviewed	*8. Silva-Valenzuela, C.A., Molina-Quiroz, R.C., Desai, P., Valenzuela, C., Porwollik, S., Zhao, M., Hoffman, R.M., Andrews-Polymenis, H., Contreras, I., Santiviago, C.A. and McClelland, M.*	Analysis of Two Complementary Single-Gene Deletion Mutant Libraries of Salmonella Typhimurium in Intraperitoneal Infection of BALB/c Mice.	***Front Microbiol***	6 : 1455 2015	US only
Published	Reviewed	*9. Elhadad, D., Desai, P., Rahav, G., McClelland, M. and Gal-Mor, O.*	Flagellin Is Required for Host Cell Invasion and Normal Salmonella Pathogenicity Island 1 Expression by Salmonella enterica Serovar Paratyphi A.	***Infect Immun***	83 : 3355-3368 2015	US only
Published	Reviewed	*10. Elhadad, D., McClelland, M., Rahav, G. and Gal-Mor, O.*	Feverlike Temperature is a Virulence Regulatory Cue Controlling the Motility and Host Cell Entry of Typhoidal Salmonella.	***J Infect Dis***	212 : 137-156 2015	US only
Published	Reviewed	*11. Marzel, A., Desai, P.T., Goren, A., Schorr, Y.I., Nissan, I., Porwollik, S., Valinsky, L., McClelland, M., Rahav, G. and Gal-Mor, O.*	Persistent Infections by Nontyphoidal Salmonella in Humans: Epidemiology and Genetics.	***Clin Infect Dis***	62 : 879-886 2016	US only
Published	Reviewed	*12. Elhadad, D., Desai, P., Grassl, G.A., McClelland, M., Rahav, G. and Gal-Mor, O.*	Differences in Host Cell Invasion and Salmonella Pathogenicity Island 1 Expression between Salmonella enterica Serovar Paratyphi A and Nontyphoidal S. Typhimurium.	***Infect Immun***	84 : 1150-1165 2016	US only
Published	Reviewed	*13. Shomer, I., Avisar, A., Desai, P., Azriel, S., Smollan, G., Belausov, N., Keller, N., Glikman, D., Maor, Y., Peretz, A.,*	Differences in Host Cell Invasion and Salmonella Pathogenicity Island 1 Expression between Salmonella enterica Serovar Paratyphi A and Nontyphoidal S. Typhimurium.	***Infect Immun***	84 : 1150-1165 2016	US only

		McClelland, M., Rahav, G. and Gal-Mor, O.				
Published	Reviewed	*Wrande M, Andrews-Polymenis H, Twedt DJ, Steele-Mortimer O, Porwollik S, McClelland M, Knodler LA.*	Genetic determinants of Salmonella enterica serovar Typhimurium proliferation in the cytosol of epithelial cells	***Infect Immun***	84 : 3517–3526 2016	US only

Appendix

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Figure and Tables

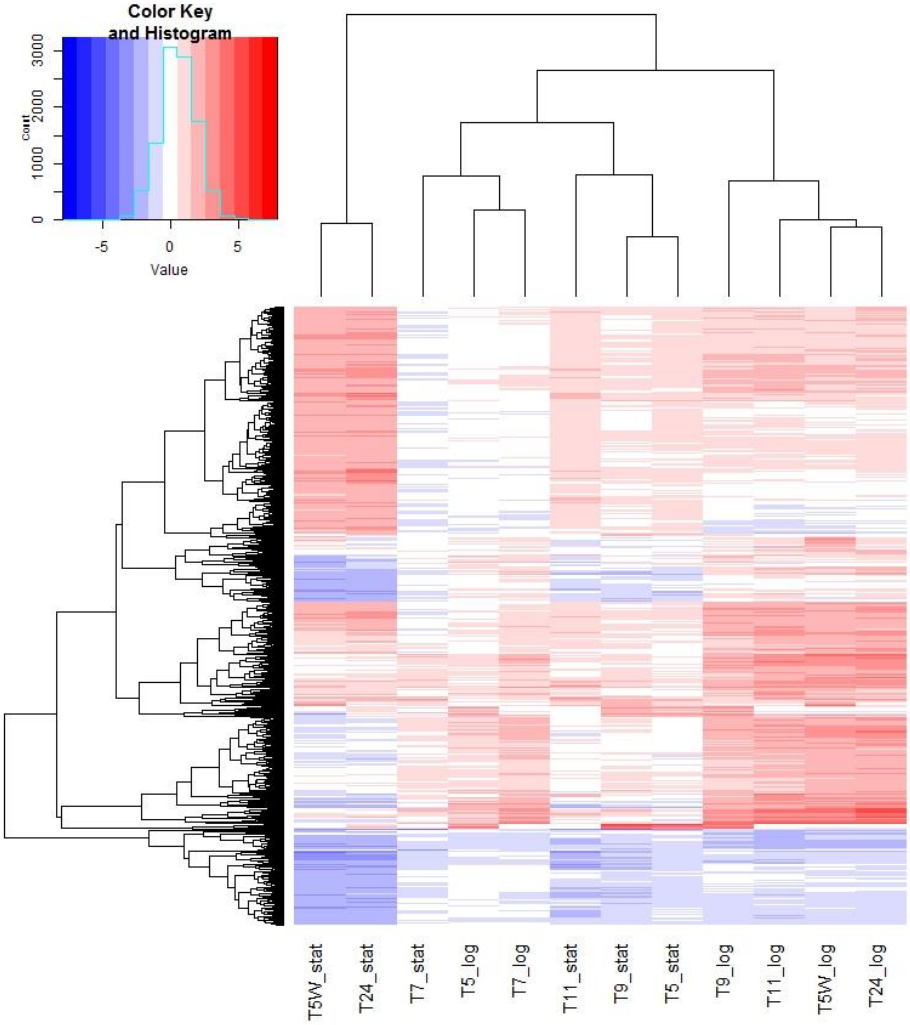

Figure 1. Heatmap of gene expression during dehydration of STm grown to logarithmic and stationary growth phases at all time.

Table 1

Table 1: Gene sets correlating with osmY expression in 915 experiments	
	False discovery rate
	OsmY: Positive correlation
Regulated by the Qse two component system	0.00
Cellular processes - Detoxification	0.00
STM2789-STM2792	0.00
STM3228-STM3231 (yqj gene cluster)	0.00
STM3534-STM3538	0.00
Cog G-carbohydrate transport and metabolism	0.00
TCA cycle variation I	0.01
Glycoside hydrolases family 13 (mostly plant sugar hydrolases)	0.01
Trehalose biosynthesis V	0.01
	OsmY: Negative correlation
Cog J-Translation	0.00
Protein synthesis - Ribosomal proteins - synthesis and modification	0.00
STM4391-STM4394	0.00
STM3419-STM3430	0.01
STM3839-STM3842	0.01
DNA metabolism - DNA replication - recombination - and repair	0.02

Table 2a: **Number of differentially expressed genes in logarithmic growth**

Library compared to T0	Number of diff genes with threshold FDR<0.001 and logFC>=2 or logFC<=-2	Uregulated genes	Downregulated genes
T5	29	29	0
T7	64	64	0
T9	135	131	4
T11	190	176	14
T24	233	229	4
T24-5W	220	213	7

Table 2b: **Number of differentially expressed genes in the stationary phase**

Library compared to T0	Number of diff genes with threshold FDR<0.001 and logFC>=2 or logFC<=-2	Uregulated genes	Downregulated genes
T5	14	13	1
T7	9	6	3
T9	32	22	10
T11	52	12	38
T24	447	317	130
T24-5W	367	208	159

Table 3. **GO enrichment analysis on every dehydration time point compared to time zero for cells in logarithmic growth.**

5 hr dehydration

Term	FDR
arginine catabolic process to glutamate	1.10E-05
arginine catabolic process to succinate	1.10E-05
succinate metabolic process	5.09E-05
arginine catabolic process	7.61E-05
glutamine family amino acid catabolic process	1.81E-04
cellular amino acid catabolic process	6.77E-04
glutamate metabolic process	8.24E-04
arginine metabolic process	2.51E-03
alpha-amino acid catabolic process	5.70E-03
carboxylic acid catabolic process	1.43E-02
organic acid catabolic process	1.43E-02
organonitrogen compound catabolic process	1.74E-02
glutamine family amino acid metabolic process	2.68E-02

7 hr dehydration

Term	FDR
arginine catabolic process to succinate	1.06E-06
arginine catabolic process to glutamate	1.06E-06
succinate metabolic process	1.45E-05
arginine catabolic process	2.86E-05
glutamine family amino acid catabolic process	1.01E-04
glutamate metabolic process	9.48E-04
organic acid catabolic process	1.70E-03
carboxylic acid catabolic process	1.70E-03

cellular amino acid catabolic process	2.10E-03
oxoacid metabolic process	2.53E-03
arginine metabolic process	2.53E-03
organic acid metabolic process	2.79E-03
alpha-amino acid catabolic process	6.94E-03
protein secretion	8.57E-03
secretion by cell	8.57E-03
secretion	9.17E-03
organonitrogen compound catabolic process	1.25E-02
carboxylic acid metabolic process	1.80E-02
small molecule catabolic process	3.79E-02
gamma-aminobutyric acid metabolic process	3.95E-02
acetate-CoA ligase activity	3.95E-02
glutamine family amino acid metabolic process	4.30E-02

9 hr dehydration

Term	FDR
arginine catabolic process to glutamate	4.28E-05
arginine catabolic process to succinate	4.28E-05
succinate metabolic process	5.72E-04
arginine catabolic process	1.12E-03
glutamine family amino acid catabolic process	3.83E-03
glutamate metabolic process	3.38E-02

24 hr dehydration

Term	FDR
transport	1.75E-04
establishment of localization	1.75E-04
arginine catabolic process to glutamate	3.54E-04
arginine catabolic process to succinate	3.54E-04
arginine metabolic process	2.55E-03
succinate metabolic process	4.32E-03
arginine catabolic process	9.64E-03
nitrogen compound transport	2.31E-02
protein secretion	2.41E-02
secretion by cell	2.41E-02
organic substance transport	2.41E-02
glutamine family amino acid catabolic process	2.41E-02
secretion	2.41E-02
membrane	4.17E-02

24 hr dehydration then 5 minutes of rehydration

Term	FDR
arginine catabolic process to glutamate	4.19E-04
arginine catabolic process to succinate	4.19E-04
arginine metabolic process	1.98E-03
succinate metabolic process	4.08E-03
arginine catabolic process	8.37E-03
glutamine family amino acid catabolic process	2.91E-02

Table 4**: Enriched GSEA categories regulated across all time points, down- and up-regulated (logarithmic growth).**

Down Category	# genes	p value	Up Category	# genes	p value
Monosaccharides	79	0.000	Central_carbohydrate_metabolism	48	0.000
Nitrogen_Metabolism	33	0.000	Pyruvate_metabolism_II:_acetyl-CoA__acetogenesis_from_pyruv	10	0.000
Nitrogen_Metabolism_-_no_subcategory	33	0.000	Phage_shock_protein_(psp)_operon	5	0.000
Capsular_and_extracellular_polysacchrides	36	0.000	Stress_Response_-_no_subcategory	17	0.001
4-Hydroxyphenylacetic_acid_catabolic_pathway	9	0.000	Stress_Response	86	0.002
L-ascorbate_utilization_(and_related_gene_clusters)	9	0.000	Type_I_secretion_system_for_aggregation	2	0.006
Nitrate_and_nitrite_ammonification	14	0.000	Protein_secretion_system__Type_I	2	0.006
Colanic_acid_biosynthesis	10	0.000	Trehalose_Biosynthesis	5	0.009
L-Arabinose_utilization	6	0.000	DNA_repair__bacterial	15	0.010
Cysteine_Biosynthesis	9	0.000	Glutathione:_Biosynthesis_and_gamma-glutamyl_cycle	3	0.013
Metabolism_of_Aromatic_Compounds	17	0.000	Osmoprotectant_ABC_transporter_YehZYXW_of_Enterobacteriale	4	0.015
Carnitine_Metabolism_in_Microorganisms	7	0.000	Arginine_and_Ornithine_Degradation	14	0.022
Siderophores	18	0.000	Glyoxylate_bypass	6	0.022
Type_VI_secretion_systems	10	0.000	Curli_production	7	0.024
Protein_secretion_system__Type_VI	10	0.000	Protein_secretion_system__Type_VIII_(Extracellular_nucleation/	7	0.024
Metabolism_of_central_aromatic_intermediates	13	0.000	Terminal_cytochrome_d_ubiquinol_oxidases	3	0.032
Nitrosative_stress	5	0.000	ATP-dependent_RNA_helicases__bacterial	4	0.035
Lysine__threonine__methionine__and_cysteine	33	0.000	Fatty_Acid_Biosynthesis_FASII	12	0.036
Formate_hydrogenase	8	0.000	F0F1-type_ATP_synthase	6	0.036
Phosphonate_metabolism	7	0.000	ATP_synthases	6	0.036
Hexose_Phosphate_Uptake_System	4	0.001	Glutamate_and_Aspartate_uptake_in_Bacteria	6	0.041
The_fimbrial_Stf_cluster	7	0.001	RecA_and_RecX	2	0.042
High_affinity_phosphate_transporter_and_control_of_PHO_regu	5	0.001	Mycobacterium_virulence_operon_involved_in_DNA_transcriptic	2	0.042
TRAP_transporters	6	0.001	Di-_and_oligosaccharides	35	0.045
Ethanolamine_utilization	17	0.001	Selenoproteins	3	0.047
Iron_acquisition_and_metabolism	21	0.001	Menaquinone_and_Phylloquinone_Biosynthesis	7	0.049

Manuscript (in revision)

OsmY Contributes to Desiccation Tolerance of *Salmonella* via Biofilm Formation

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A short title: OsmY enhances Salmonella tolerance to desiccation

Abstract:

Desiccation tolerance is an essential feature enabling the survival of human pathogens outside the host. The ability of the foodborne pathogen *Salmonella enterica* to tolerate dehydration is demonstrated by outbreaks related to consumption of dry food and its long-term persistence in agricultural soil. Despite the importance of desiccation tolerance, knowledge regarding the survival mechanisms of *Salmonella* in dry environments is still scarce. In the present study we demonstrate that desiccation tolerance in *S. enterica* sv Typhimurium is enhanced by the release of OsmY, an osmotically-induced protein of yet unknown function. OsmY is released during dehydration and contributes to bacterial attachment, co-aggregation and biofilm formation. Biofilms were previously shown to confer tolerance against a variety of stresses, including desiccation; however, this is the first report demonstrating the development of biofilm as an adaptation response of dehydrating Salmonella and assigning a specific role for OsmY in this process.

Keywords:

Salmonella, dehydration, desiccation, stress, biofilm, osmY

Significance statement:

Tolerance to dehydration is an important trait allowing survival of pathogens in the dry environment outside the host. The foodborne pathogen *Salmonella enterica* was reported to persist for long periods of times in agricultural soils and is implicated also in salmonellosis outbreaks related to dry food. Despite the importance of dehydration tolerance, knowledge regarding the survival mechanisms of *Salmonella* in dry environments is still scarce. In the present study we demonstrate that OsmY, an osmotically-induced protein is released by *S. enterica* during dehydration and increases bacterial survival by enhancing attachment to surface, co-aggregation and biofilm formation. While biofilms are known to confer tolerance against a variety of stresses, including dehydration, this is the first report demonstrating the development of biofilm in response to dehydration and assigning a novel functional role to OsmY in this process.

Introduction

More than 1 million episodes of foodborne illness in the United States are caused each year by nontyphoidal *Salmonella enterica* serotypes. Among illnesses caused by foodborne pathogens, *S. enterica* serotypes are the leading cause of hospitalizations and death (1). *Salmonella* is a zoonotic foodborne pathogen that colonizes the intestinal tracts of multiple hosts, including humans and numerous mammals, poultry, and reptiles. Once excreted from the host, *Salmonella* faces a hostile environment characterized by limited nutrient availability, osmotic stress, large variations in temperature and pH, and dehydration (2).

The ability of the pathogen to survive in the food chain depends greatly upon its ability to respond effectively to environmental changes (3). Dehydration is one of the critical stresses *Salmonella* may frequently encounter. *Salmonella* can survive from several weeks to several years on dry surfaces (4, 5), dry soil (6) or in low moisture food (7).

Reduction of water content in food products is often used in the food industry as a method for limiting growth of both foodborne pathogens and spoilage microorganisms. However, numerous low a_w food products, such as peanuts, peanut butter, chocolate, and powdered infant formula, have been implicated in the last decade as vehicles for *Salmonella* to cause large national and international salmonellosis outbreaks (8). Just recently, a large recall was announced in Israel, due to contamination of cereals (cornflakes and others) by *Salmonella* (NEW RF) The response of *Salmonella* to desiccation as well as to other environmental stresses was recently reviewed (9-) Add our review. In order to adapt to water loss, bacteria use a variety of sensors, signal-transduction systems, alternative sigma factors and transcriptional regulators, which result in an adaptive physiological response aimed at increasing cellular concentration of compatible solutes. In recent years, transcriptomic analysis was employed in order to identify *Salmonella* genes potentially involved in dehydration (10-15). While these studies have advanced our knowledge regarding the desiccation stress response, the exact mechanisms that render *Salmonella* tolerant to desiccation remain unclear.

Results

Upon desiccation, *Salmonella* releases a factor that enhances desiccation tolerance.

We recently reported that *Salmonella enterica* serotype Typhimurium (STm) dehydration tolerance and long-term persistence are influenced by various growth- and dehydration conditions, such as temperature, pH, growth phase, type of growth medium, dehydration rate, and the presence of various exogenous compounds (4). In order to examine the possible effect of cell-density on bacterial persistence, we have compared the survival of high-density (10^7-10^9 CFU/ml) and low-density populations (10^4-10^6 CFU/ml) during dehydration and subsequent long-term dry storage at 4˚C (Fig. 1). The percentage of surviving bacteria following 22 h dehydration ranged from 48% for high density populations (10^9 CFU/ml) to 2% at low density populations (10^3 CFU/ml) (Fig. 1A). Subsequent cold storage (4˚C) of high density dried cells resulted in a 10-fold CFU reduction within the first two weeks and no further change in the number of viable cells for the next three months. In contrast, low density populations rapidly declined and no survivors were detected after four weeks of cold storage (Fig. 1B). The observed results were reminiscent of a quorum-sensing response, where the behavior of the entire population is determined by cell density. Quorum-sensing bacteria produce and release chemical signal molecules called autoinducers that increase in concentration as a function of cell density (16). In order to determine if the tolerant phenotype of high-density STm was mediated by autoinducers, dense (10^9 CFU/ml) STm cells suspended in 50 µl of sterile double-distilled water (SDDW), were dried in a 96-well plate and then rehydrated with SDDW to their initial volume (50 µl), as described in Materials & Methods. The suspension was filtered through a 0.22 µm membrane in order to separate potential autoinducers from the bacterial suspension. Similarly, a control filtrate was prepared from non-dehydrated bacteria (at the same concentration) that were incubated in SDDW for a comparable period of time and temperature (22 h, at 25˚C). The two filtrates were added to low-density STm cells, which underwent 22 h dehydration period followed by cold-storage for 4 weeks. The desiccated cell filtrate (DCF), but not a non-desiccated cell filtrate (NDCF), derived from bacteria suspended in SDDW, increased desiccation tolerance of low-density STm, suggesting that high-density cells release a diffusible molecule, which may account for the observed effect (Fig. 2).

<u>OsmY protein increases desiccation tolerance</u>.

In order to uncover the nature of this potentially diffusible factor, we tested the effect of heat and proteinase K treatments on the filtrate (DCF) activity. Both treatments significantly decreased the effect of the filtrate (Fig. 2), suggesting the active compound in DCF to be a protein. In order to further characterize the putative compound(s), designated *Salmonella* desiccation-response factor

(SDRF), we prepared a concentrated filtrate and size-separated it by Fast Performance Liquid Chromatography (FPLC), and tested the eluted fractions for SDRF activity, as described for DCF (Fig. 3). Fractions possessing the highest activity were trypsinized and subjected to amino-acid sequencing. All fractions with high activity contained peptides derived from OsmY (STM4561 in STm LT2, SL1344_4489 in STm SL1344), a stress-response protein, which was originally identified in *Escherichia coli* about two decades ago as an osmotic stress-inducible gene (17) and as a carbon starvation-inducible gene (18). The expression of the *osmY* gene is under the control of the global stress-response sigma factor RpoS (19, 20) and is negatively regulated by the global regulators Lrp, the cyclic AMP receptor protein-cAMP complex, and the integration host factor (19). The STm 1344 *osmY* gene contains 205 amino acids (http://www.kegg.jp/dbget-bin/www_bget?sey:SL1344_4489) with a putative leader peptide of 28 amino acids (Phobius; http://phobius.sbc.su.se/). Orthologs of the *osmY* gene (≥ 30 % sequence identity, $p < 1e\text{-}20$) are widely conserved across proteobacteria and are present in most *Enterobacteriaceae*. Homologs of *osmY* are highly conserved across all *Salmonella* indicating that this gene arose quite early during evolution. The gene is intact in all host-adapted serovars of *Salmonella* examined (Typhi, Paratyphi A, Sendai, Paratyphi C, Choleraesuis, Dublin and Gallinarum), and might therefore be important in the life cycle of *Salmonella*.

To test whether OsmY is responsible for the DCF activity, an *osmY* knockout mutant was generated and tested for desiccation tolerance. Dried *osmY* mutant cells were more susceptible to dehydration and subsequent long-term cold storage, compared to wild-type (WT) cells (Fig. 4A), and did not produce SDRF (Fig. 4B). Genetic complementation restored the WT phenotype, including secretion of active SDRF, as well as long-term persistence at 4˚C. An *osmY* mutant transformed with the vector alone (pBR322, control) retained the mutant's phenotype (Fig. 4).

To further confirm that OsmY is indeed the active component of SDRF, we generated a recombinant OsmY (rOsmY-6His) protein and tested its activity. Recombinant OsmY displayed a SDRF-like activity on dehydration tolerance in a dose-dependent manner. The highest protective activity (31% survival) was obtained at a concentration of 10 µg/ml, and the effect faded at higher protein concentrations up to 100 µg/ml, where the activity was severely diminished (Fig. 5A). As a control, we also tested the effect of bovine serum albumin (BSA), an unrelated protein. At a comparable concentration (10 µg/ml), BSA had a protective effect compared to SDDW alone, but it was much lower than the rOsmY-mediated effect. Unlike rOsmY, BSA's protective activity

increased gradually with increasing concentration, and in concentration of 100 µg/ml yielded 24% survival (Fig. 5A). The effect of rOsmY and BSA on long-term persistence of dried cells in cold storage (4˚C) was also examined at 10 and 100 µg/ml. Exogenous rOsmY, at 10 µg/ml, enhanced STm persistence during storage to 22.4% survival, while BSA did not display a significant effect at the tested concentrations (10 and 100 µg/ml), compared to SDDW (Fig. 5B), and percentage of survival remained under 2%. These findings suggest that rOsmY contributes to STm persistence both during dehydration as well as during subsequent cold-storage.

The OsmY protein promotes biofilm formation during dehydration.

OsmY is considered a stress response protein, but its precise function is still unknown. Viability staining of the cells after dehydration (Fig. 6A) demonstrated the presence of massive cell aggregates containing viable (green) cells. During subsequent cold-storage (Fig. 6B), the number of dead cells (red) increased, however, cells residing within the aggregates tended to be more protected against desiccation stress compared to individual cells (Fig. 6B). Quantification of the number of cell aggregates after dehydration by confocal laser scanning microscopy demonstrated the presence of high numbers of aggregates in the WT bacteria, and significantly lower number of aggregates in the *osmY*⁻ mutant. Genetic and phenotypic complementation restored the level of the aggregates to that of the WT strain (Fig. 6C). Microbial aggregation may represent a developmental stage during biofilm formation, a unique growth mode, which serves as a universal survival strategy against multiple environmental stresses (21). Biofilm bacteria are usually attached to each other and to a surface and are embedded within an extracellular polymeric substance (EPS) (22). It is likely that during dehydration, STm senses changes in the environment and responds by initiating biofilm formation, which may renders the cells tolerant to the water-less environment. In order to test this hypothesis, we further examined the biofilm mass formed during dehydration of SDDW-suspended WT, *osmY*⁻ mutant and *osmY*⁺ strains using the crystal-violet biofilm-formation assay. Both WT and *osmY*-expressing strains developed high amounts of biofilm after 8-22 h compared to the *osmY*⁻ mutant strain (Fig. 6D). The biofilm mass of the WT strain in fully dehydrated cells (i.e., after 22 h) was 1.7-fold higher compared to that of the *osmY*⁻ mutant. Genetic complementation or addition of exogenous rOsmY restored the biofilm mass (Fig. 6D).

The extracellular proteins in the WT's filtrate were characterized by Liquid chromatography tandem-mass spectrometry (LC-MS/MS) to confirm that native OsmY protein is released by STm during

dehydration. OsmY was detected in the filtrate of dehydrating bacteria after 8 h (Figure S2) with a relative abundance of around 4%. The relative abundance increased up to 11% of the total extracellular proteins at 22 h. Together with the fact that an *osmY*⁻ mutant showed much lower biofilm production, these findings support the notion that native OsmY protein released during dehydration is functionally associated with biofilm formation.

Scanning electron microscopy (SEM) demonstrated that the majority of the WT cells as well as the complemented *osmY*⁻ cells (*osmY*⁺) were located in large aggregates. Frequently, these aggregates were coated by an EPS. Thin fimbriae-like structures connected the cells within each cluster. In contrast, *osmY*⁻ cells did not form large clusters and were devoid of any observable EPS (Fig. 7A). Higher magnification imaging of individual cells revealed that WT but not *osmY*⁻ mutant cells appeared to be coated by rough EPS material. Genetic complementation of the *osmY*⁻ mutant restored the WT phenotype (Fig. 7B). It is likely, that OsmY may act as an integral component of the surface-associated matrix or capsular material in desiccation-stressed *Salmonella*. Indeed, addition of rOsmY to low density cells of the WT and *osmY* knockout mutant strains resulted in the formation of a rough, capsular-like envelope surrounding the cells (Fig. 7C).

Production of known EPS matrix components was previously shown to play a significant role in *Salmonella* resistance to desiccation (23-25). While those studies examined the role of pre-formed biofilm in protecting the cells from desiccation stress, our findings are the first to demonstrate the induction of biofilm formation in response to desiccation stress. *Salmonella* may sense dehydration and respond by increasing the extracellular concentration of OsmY, which is likely to facilitate formation of biofilm as a novel defense mechanism. The similar effects imposed by the WT and the exogenous rOsmy (phenotypic complementation) suggest that OsmY may have a structural function, likely enhancing cell-cell interactions and biofilm formation.

Besides osmotic stress and nutrient starvation, OsmY expression was also linked to exposure of bacteria to other stressors, such as carbon starvation (ref), bile salts (26) and cationic peptides (27). Analysis of available transcriptomic data in STm (28) demonstrates that *osmY* is co-regulated with genes required for proline/glycine betaine transport systems and biosynthesis of trehalose (Table S1), supporting previous reports on the expression of *osmY* during osmotic stress and our findings regarding its role in desiccation tolerance. Most prominently, expression of the *osmY* gene is

strongly correlated to genes that are negatively-regulated by the PreA/PreB (QseB/QseC) two-component system under a variety of stress and growth conditions (29) (Table S1).

Bioinformatic analysis revealed that OsmY possesses two conserved BON (Bacterial OsmY and Nodulation) sites with a putative binding domain. It was proposed that the two domains interact with the inner- and outer membranes of the bacterium thus stabilizing their structure by preventing shrinkage of the inner membrane during water-loss (30). However, recent studies reported that OsmY is secreted into the growth medium during stationary phase (31), suggesting that OsmY may have an additional function. Our findings regarding the role of OsmY in bacterial co-aggregation and biofilm formation offer a novel function for this widespread protein and are consistent with previous studies regarding the protective role of biofilm against environmental stresses, including desiccation, in both *Salmonella* as well as in other bacterial species (21, 22).

Materials & Methods

Inoculum preparation and growth conditions. *Salmonella enterica* serovar Typhimurium SL1344 was used in this study. Bacteria were grown on xylose lysine deoxycholate (XLD) agar (Difco Laboratories; Sparks, MD). A freshly isolated bacterial colony was spread on LB-agar (Difco) with a sterile swab and incubated for 20 h at 37^{o}C. Cells were harvested by resuspension in sterile double deionized water (SDDW) using a sterile rubber policeman, washed three times in SDDW (3800g x 5 min) at room temperature, and the final pellet was resuspended in SDDW to the required concentration.

Dehydration conditions and survival assay. Fifty microliter of bacteria containing 10^3-10^8 CFU were dehydrated in 96-well polystyrene plates (Greiner Bio-One; Frickenhausen, Germany) for 22 h at 25^{o}C and 40% relative humidity (RH) in a climate-controlled incubator (Climacell, MMM Group; Munich, Germany). Viable counts at time zero (before dehydration) and following dehydration were determined, as described previously (32). Dehydration survival was calculated as percentage of viable bacteria after 22 h or after 4 weeks following storage of the dried cells at 4^{o}C, 40-45% RH).

Filtrate preparations and manipulations. A filtrate was prepared from dried cells as follows: STm cells (10^8 CFU/well in 50µl) were dried for 22 h as described above. The dried cells were rehydrated with the initial volume of SDDW and the bacterial suspension was spun down (3,600 g,

5 min) and the supernatant was filtered through a 0.22 membrane (Sartorius Stedim Biothech; Goettingen, Germany) to remove bacterial cells and large debris. Similarly, a control filtrate was prepared from non-desiccated cells incubated in SDDW for 22 h at 25˚C. In some cases, the filtrate was boiled for 10 min or treated with 100 µg/ml of proteinase K (Sigma-Aldrich; St. Louis, MO) for 1 h at 50^{o}C. The filtrate was either stored at -20^{o}C, or lyophilized and stored at 4^{o}C.

Protective activity assay. *Salmonella* cells at a final concentration of 2x10^{5} CFU/ml SDDW were dehydrated alone (control) or in the presence of various filtrates, filtrate's fractions, rOsmY or Bovine serum albumin (BSA). The bacteria were dehydrated in 96-well polystyrene plates for 22 h and subsequently stored for 4 weeks at 4˚C, as described above. The number of surviving bacteria was determined immediately after dehydration (22 h) and once a week during 4 weeks of storage.

Purification of the active component of SDRF by Fast Performance Liquid Chromatography (FPLC). For further characterization of SDRF, a crude filtrate was prepared from a 150 ml bacterial suspension following 22 h dehydration on empty petri dishes (90 mm Miniplast; Ein-Shemer, Israel). The dried cells were resuspended in SDDW, centrifuged for 10 min. at 3,600 g and then filtered through a 0.22 µm membrane to remove large bacterial debris. The filtrate was lyophilized and kept at 4˚C. A 50-fold concentrated crude filtrate was prepared by resuspending the dry material in 3 ml DDW and was separated by gel filtration using FPLC (ÄKTAexplorer; GE Healthcare) at the ARO core protein facility unit. Separation was performed in double-distilled water at 4ºC and flow rate of 1 ml/min, on a dextran prepacked column Superdex 75, enabling size fractionation from 3000 to 600,000 kDa (GE Healthcare; Uppsala, Sweden). Fractions were eluted in 150 ml of SDDW. In total, 100 fractions of 1.5 ml each were eluted and the SDRF activity of each fraction was tested, as described above.

Protein sequencing. Active FPLC-eluted fractions were sent to the Smoler Proteomics Center (Technion, Haifa, Israel) for trypsinization and amino acid sequencing by LC-MS/MS on an Orbitrap XL mass spectrometer (Thermo Scientific; San Jose, CA). Peptides were identified by the Discoverer software (Oracle) against the *Salmonella* NCBI-nr database, and a decoy database (in order to determine the false discovery rate). All identified peptides were filtered with at least medium confidence, top rank, mass accuracy and a minimum of 2 peptides. High confidence peptides passed the 1% FDR (false discovery rate) threshold. Medium confidence peptides passed the 5% FDR threshold. Relative abundance of the target protein was determined by calculating the ratio between the target protein peak area to the peak area of total protein in the sample.

Expression of recombinant OsmY. An *osmY* copy from WT strain was amplified by PCR using primers osmYpet28-For and osmYpet28-Rev (Table S2), cut with restriction enzymes (FastDigest; Thermoscientific, Vilnuis, Lithuania) NcoI and SalI according to manufacturer instructions, and cloned into the pET28/His vector pre-cut with the same enzymes using T4 DNA ligase (Thermo Scientific). The construct was transformed into competent *E. coli* BL21 by electroporation. Bacteria were grown in LB broth at 37^{o}C to an O.D$_{600}$ of 0.6. Expression of recombinant protein was induced by addition of 2 mM IPTG (Sigma Aldrich) and the cells were further incubated at 37^{o}C for 3 h. Harvested bacteria were lysed by sonication in a lysis buffer (300 mM KCl, 50mM KH_2PO_4 and 5mM imidazole; pH 8), and the lysate was centrifuged at 8000xg for 20 min. The lysate was further cleared by filtration through a 0.22 µm membrane and 5 ml portions were subsequently applied to Bio-Scale^{TM} Mini Profinity^{TM} IMAC Cartridges (Bio-Rad; Hercules, CA). The recombinant OsmY protein was purified according to the manufacturer's instructions (BioRad), and the presence of a single protein band representing the purified recombinant protein was confirmed by SDS-PAGE. The recombinant protein was concentrated and desalted using Vivaspin6 3kDa columns (GE Healthcare). The protein concentration was determined using the BCA^{TM} Protein Assay Kit (Thermo Scientific) and the protein was stored at -20^{o}C.

Target deletion and genetic complementation of osmY gene. Target deletion mutation was generated by the lambda-red recombinase procedure (33), using primers listed in Table S2, with the pKD4 plasmid carrying a Kan^{R} cassette as template for PCR reactions. Mutations were confirmed using nearby locus-specific primers (Table S2) with the respective primers k2 or kt (33), and verified by sequencing. Genetic complementation was performed by introducing a WT copy of the *osmY* gene into the Δ*osmY* STm mutant using the pBR322 cloning vector. Briefly, an *osmY* copy from the WT was PCR-amplified using osmYpbr322-For and osmYpbr322-Rev primers (Table S2), cut with NheI and SalI (FastDigest; Thermoscientific), cloned into pBR322 plasmid as described above and electroporated into competent Δ*osmY* mutant cells. A mutant transformed with pBR322 vector alone (no insert) served as a control in desiccation tolerance experiments.

Microscopic observations. For confocal microscopy observations, dried bacteria (10^8 CFU/well) were resuspended in 30 µl of staining solution (LIVE/DEAD® *Bac*Light™

bacterial viability kit; Molecular Probes; Eugene, Oregon) and stained according to the manufacturer's instructions. The cells were transferred to glass slides and visualized under a confocal laser-scanning microscope (Olympus IX81, Tokyo, Japan). For scanning electron microscopy a bacterial suspension (50 µl) containing 10^8 or 10^5 CFU/well were desiccated on plastic discs (5 mm diameter) which had been placed at the bottom of the wells of a 96-well polystyrene plate. Following dehydration, bacteria were fixed using glutaraldehyde (4% in phosphate buffer 0.1M pH 7.2) for 1h at room temperature. The discs were rinsed 5 times for 10 min with phosphate buffer, and then dehydrated in increasing ethanol concentrations (25, 50, 75 and 95%) for 20 minutes for each concentration. Finally, discs were washed 4 times for 30 min in 100% ethanol, dried and coated with gold-palladium using Polaron SC7640 Spatter coater (Quorum Technologies; Newhaven, UK). The discs were then visualized using the extra high resolution scanning electron microscope Magellan^{TM} 400L (FEI Company; Hillsboro, Oregon).

Enumeration of bacterial aggregates. Dried high-density *Salmonella* cells were treated as described above and visualized under a confocal laser-scanning microscope. The number of bacterial aggregates was calculated under magnitude x400 in 50 microscopic fields per replicate. Bacterial clusters were considered as aggregates if they contained more than ten bacterial cells.

Biofilm formation assay. High-density bacteria (10^8 CFU/well) were desiccated, as described above, and biofilm formation was tested using the microtiter dish biofilm formation assay (34), following 5, 8, 16 and 22 hours of dehydration.

Statistical analysis. All experiments were repeated at different days at least twice. Each experiment was performed in triplicates. Comparison of percentage of bacterial survival was performed by One-way analysis of variance (ANOVA) and Tukey-Kramer Multiple Comparisons Test using the GraphPad Instat3 software (GraphPad Software Inc.; La Jolla, CA). Differences between means were considered significant at $P < 0.05$.

References

1. Scallan E, *et al.* (2011) Foodborne illness acquired in the United States-major pathogens. *Emerg Infect Dis* 17(1):7-15.
2. Winfield MD & Groisman EA (2003) Role of nonhost environments in the lifestyles of *Salmonella* and *Escherichia coli*. *Appl Environ Microb* 69(7):3687-3694.
3. Humphrey T (2004) *Salmonella*, stress responses and food safety. *Nat Rev Microbiol* 2(6):504-509.
4. Gruzdev N, Pinto R, & Sela S (2012) Persistence of *Salmonella enterica* during dehydration and subsequent cold storage. *Food Microbiol* 32(2):415-422.
5. Hiramatsu R, Matsumoto M, Sakae K, & Miyazaki Y (2005) Ability of Shiga toxin-producing *Escherichia coli* and *Salmonella* spp. to survive in a desiccation model system and in dry foods. *Appl Environ Microb* 71(11):6657-6663.
6. Bernstein N, Sela S, & Neder-Lavon S (2007) Effect of irrigation regimes on persistence of *Salmonella enterica* serovar Newport in small experimental pots designed for plant cultivation. *Irrigation Sci* 26(1):1-8.
7. Podolak R, Enache E, Stone W, Black DG, & Elliott PH (2010) Sources and risk factors for contamination, survival, persistence, and heat resistance of *Salmonella* in low-moisture foods. *J Food Protect* 73(10):1919-1936.
8. Beuchat LR, *et al.* (2013) Low-water activity foods: Increased concern as vehicles of foodborne pathogens. *J Food Protect* 76(1):150-172.
9. Spector MP & Kenyon WJ (2012) Resistance and survival strategies of *Salmonella enterica* to environmental stresses. *Food Res Int* 45(2):455-481.
10. Huang Y, Leming CL, Suyemoto M, & Altier C (2007) Genome-wide screen of *Salmonella* genes expressed during infection in pigs, using in vivo expression technology. *Appl Environ Microb* 73(23):7522-7530.
11. Deng XY, Li ZX, & Zhang W (2012) Transcriptome sequencing of *Salmonella enterica* serovar Enteritidis under desiccation and starvation stress in peanut oil. *Food Microbiol* 30(1):311-315.
12. Gruzdev N, *et al.* (2012) Global transcriptional analysis of dehydrated *Salmonella enterica* serovar Typhimurium. *Appl Environ Microb* 78(22):7866-7875.
13. Finn S, *et al.* (2013) ProP is required for the survival of desiccated *Salmonella enterica* serovar Typhimurium cells on a stainless steel surface. *Appl Environ Microb* 79(14):4376-4384.
14. Finn S, Condell O, McClure P, Amézquita A, & Fanning S (2013) Mechanisms of survival, responses and sources of *Salmonella* in low-moisture environments. *Frontiers in Microbiology* 4:331.

15. Li HP, *et al.* (2014) Effect of the local microenvironment on survival and thermal inactivation of *Salmonella* in low- and intermediate-moisture multi-ingredient foods. *J Food Protect* 77(1):67-74.

16. Miller MB & Bassler BL (2001) Quorum sensing in bacteria. *Annu Rev Microbiol* 55:165-199.

17. Yim HH & Villarejo M (1992) *osmY*, a new hyperosmotically inducible gene, encodes a periplasmic protein in *Escherichia coli*. *J Bacteriol* 174(11):3637-3644.

18. Hengge-Aronis R (1993) Survival of hunger and stress: The role of *rpoS* in early stationary phase gene regulation in *E. coli*. *Cell* 72(2):165-168.

19. Lange R, Barth M, & Henggearonis R (1993) Complex transcriptional control of the sigma(S)-dependent stationary-phase-induced and osmotically regulated *osmY* (Csi-5) gene suggests novel roles for Lrp, cyclic-AMP (cAMP) receptor protein-cAMP complex, and ntegration host factor in the stationary-phase response of *Escherichia coli*. *J Bacteriol* 175(24):7910-7917.

20. Yim HH, Brems RL, & Villarejo M (1994) Molecular characterization of the promoter of OsmY, an RpoS-dependent gene. *J Bacteriol* 176(1):100-107.

21. Hall-Stoodley L, Costerton JW, & Stoodley P (2004) Bacterial biofilms: From the natural environment to infectious diseases. *Nat Rev Microbiol* 2(2):95-108.

22. Costerton JW, Stewart PS, & Greenberg EP (1999) Bacterial biofilms: A common cause of persistent infections. *Science* 284(5418):1318-1322.

23. Garmiri P, Coles KE, Humphrey TJ, & Cogan TA (2008) Role of outer membrane lipopolysaccharides in the protection of *Salmonella enterica* serovar Typhimurium from desiccation damage. *FEMS Microbiology Letters* 281(2):155-159.

24. Gibson DL, *et al.* (2006) *Salmonella* produces an O-antigen capsule regulated by AgfD and important for environmental persistence. *J Bacteriol* 188(22):7722-7730.

25. White AP, Gibson DL, Kim W, Kay WW, & Surette MG (2006) Thin aggregative fimbriae and cellulose enhance long-term survival and persistence of *Salmonella*. *J Bacteriol* 188(9):3219-3227.

26. Bernstein C, Bernstein H, Payne CM, Beard SE, & Schneider J (1999) Bile salt activation of stress response promoters in *Escherichia coli*. *Curr Microbiol* 39(2):68-72.

27. Oh JT, *et al.* (2000) Cationic peptide antimicrobials induce selective transcription of *micF* and *osmY* in *Escherichia coli*. *Bba-Biomembranes* 1463(1):43-54.

28. Meysman P, *et al.* (2014) COLOMBOS v2.0: an ever expanding collection of bacterial expression compendia. *Nucleic Acids Res* 42(D1):D649-D653.

29. Merighi M, *et al.* (2009) Genome-wide analysis of the PreA/PreB (QseB/QseC) regulon of *Salmonella enterica* serovar Typhimurium. *BMC Microbiology* 9(1):42.

30. Yeats C & Bateman A (2003) The BON domain: a putative membrane-binding domain. *Trends Biochem Sci* 28(7):352-355.

31. Kotzsch A, *et al.* (2011) A secretory system for bacterial production of high-profile protein targets. *Protein Sci* 20(3):597-609.

32. Gruzdev N, Pinto R, & Sela S (2011) Effect of desiccation on tolerance of *Salmonella enterica* to multiple stresses. *Appl Environ Microb* 77(5):1667-1673.

33. Datsenko K & Wanner B (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 97:6640 - 6645.

34. Merritt JH, Kadouri DE, & O'Toole GA (2005) Growing and analyzing static biofilms. *Current protocols in microbiology*:1B. 1.1-1B. 1.18.

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Figures

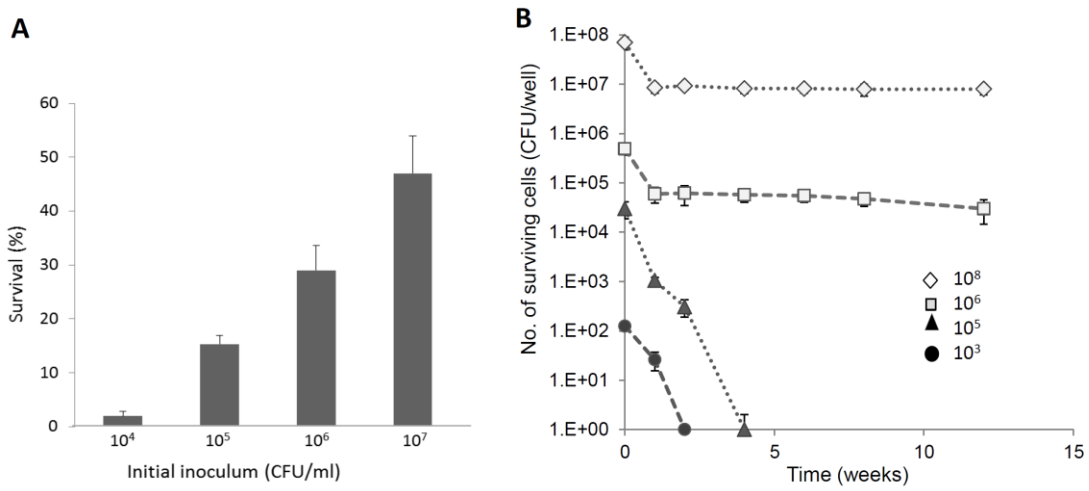

Fig. 1. Effect of population density on desiccation tolerance.

Salmonella cells suspended in SDDW (50 µl) were dehydrated for 22 h at 40% RH in 96-well plate at concentration of 10^3-10^8 CFU/well and then stored for 4 weeks at 4°C. (A) Bacterial survival at 22 h is expressed as mean percentage of viable bacteria compared to the number of bacteria at time zero (before desiccation), which was considered 100%. (B) Bacterial persistence during subsequent cold storage is presented as mean CFU/well. Data shown represent the mean of three independent experiments, each performed in triplicate. Error bars denote the standard deviation of the mean.

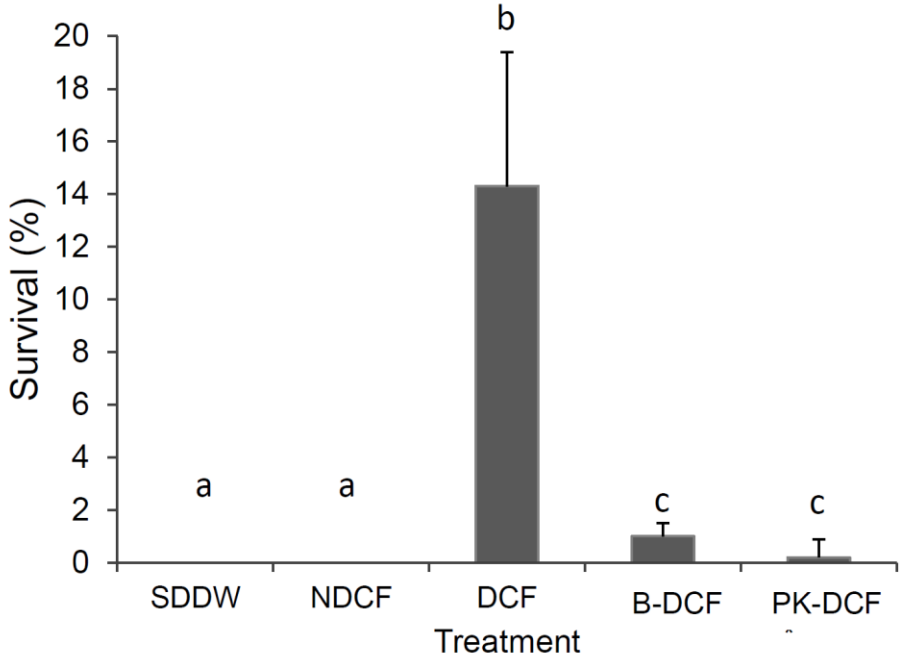

Fig. 2. High-density dehydrated cells release a factor which increases desiccation tolerance of low-density cells.

Dehydrated cells' filtrate (DCF) recovered from high-density dehydrated cells increases dehydration tolerance of low-density cells. Survival was calculated as percentage of viable bacteria after 4 weeks of cold storage; the number of bacteria at time zero (before desiccation) was considered 100%. SDDW, sterile double-distilled water; NDCF, non-dehydrated cells' filtrate; B-DCF, boiled DCF; PK-DCF, Proteinase K-treated DCF. Data shown represent the mean of three independent experiments, each performed in triplicate. Error bars denote the standard deviation of the mean. Bars marked with different letters indicate significant difference (One-way ANOVA, $P < 0.05$) in survival between treatment regimens.

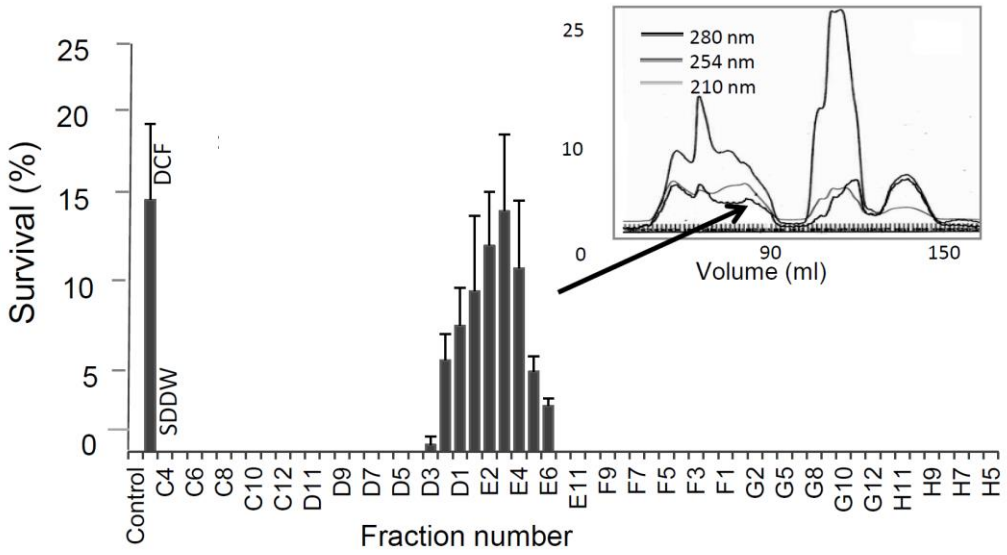

Fig. 3. Analysis of FPLC-eluted fractions on desiccation tolerance.

Crude cell filtrate (DCF) prepared from high-density dehydrated cells was fractionated by FPLC and the protective activity of each fraction on desiccation tolerance of low-density cells (10^4 CFU/well) was assayed. DCF served as a positive control and SDDW served as negative control. A chromatogram representing the absorbance of various fractions at 280, 254, and 210 nm, is shown in the inset. Data shown represent the mean of two independent experiments, each performed in triplicate. Error bars denote the standard deviation of the mean.

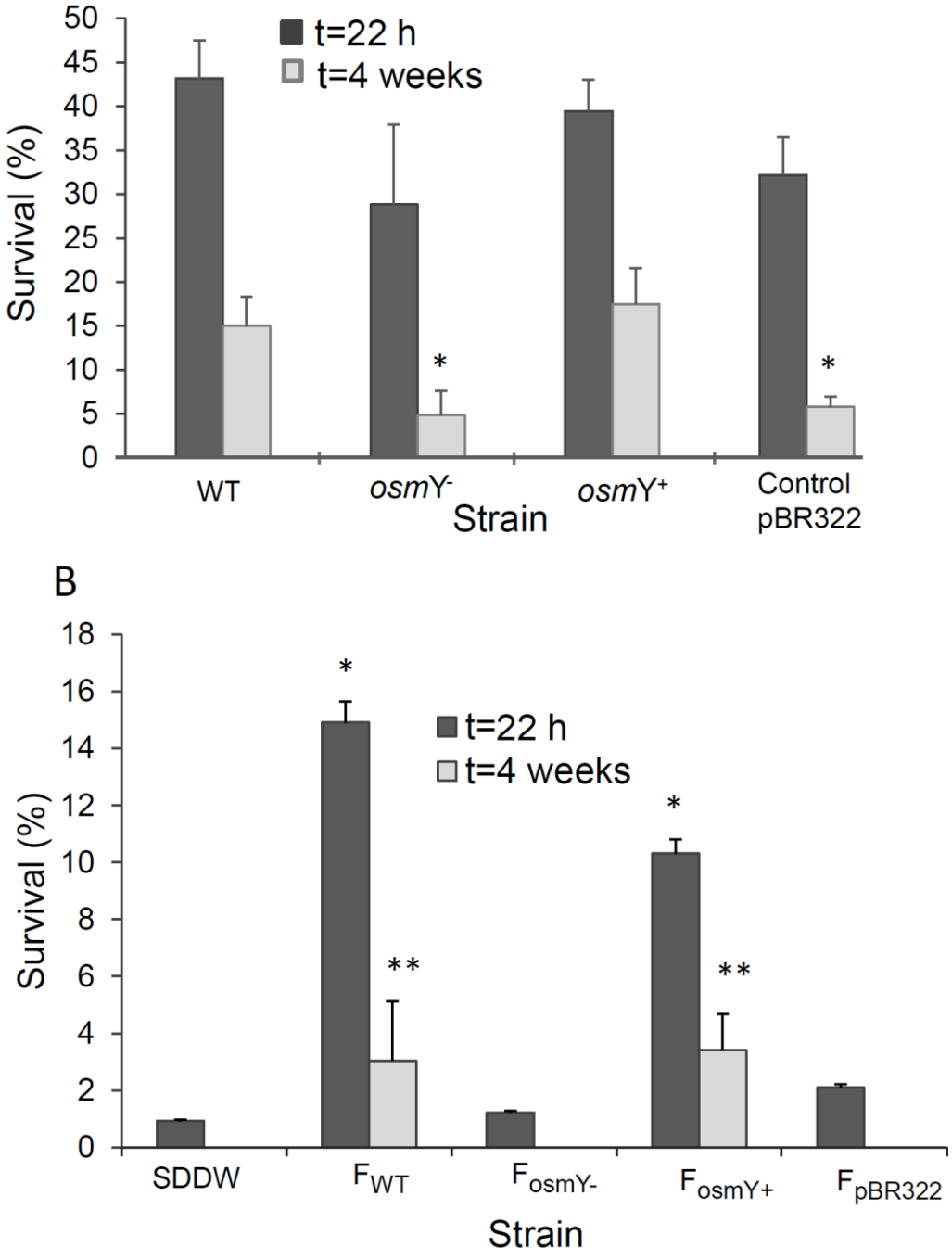

Fig. 4. Genetic and phenotypic characterization of OsmY. (A) Survival of dehydrated STm strains (10^8 CFU/well) immediately after dehydration at 25°C (t=22 h) and following 4 weeks of cold-storage (t=4 weeks). WT, wild type strain; *osmY*$^-$, Δ*osmY* mutant, *osmY*$^+$, Δ*osmY*$^-$ mutant complemented with pBR322 carrying the WT *osm*Y gene; Control pBR322, Δ*osmY*$^-$ mutant containing the vector alone (pBR322). **(B)** Filtrates from WT (F_{WT}), the *osmY*$^-$ mutant (F_{osmY^-}), the *osm*Y$^-$ mutant complemented with the WT gene (F_{osmY^+}) and filtrate from the *osmY*$^-$ mutant transformed with pBR322 vector alone (F_{pBR322}) were tested for SDRF protecting activity utilizing low density STm cells (10^4 CFU/well) after 22 h and 4 weeks, as described above. Bars marked with asterisks

indicate significant difference (One-way ANOVA, $P < 0.05$) in survival between the different strains and the WT (A), or between different filtrates and the SDDW control (B) at 22 h (*) and 4 weeks (**), respectively.

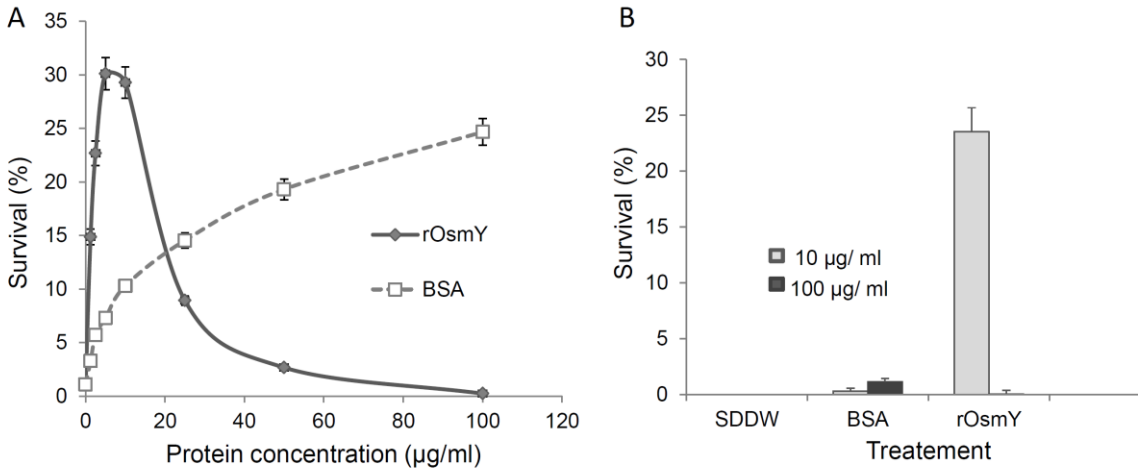

Fig. 5. Effect of exogenous rOsmY protein on desiccation survival.

(A) Effect of increasing concentrations of exogenous rOsmY and an unrelated protein (BSA) on survival of low-density STm immediately following dehydration (t=22 h). **(B)** Effect of exogenous rOsmY and BSA on survival of low-density STm following 4-weeks of cold-storage. STm cells were dehydrated in the presence of rOsmY, SDDW or BSA and the survival was determined following dehydration. The survival (percentage) is presented as the ratio of viable bacteria to the initial number of bacteria before dehydration. Data represent the mean of three independent experiments, each performed in triplicate. Error bars denote the standard deviation of the mean (n=9).

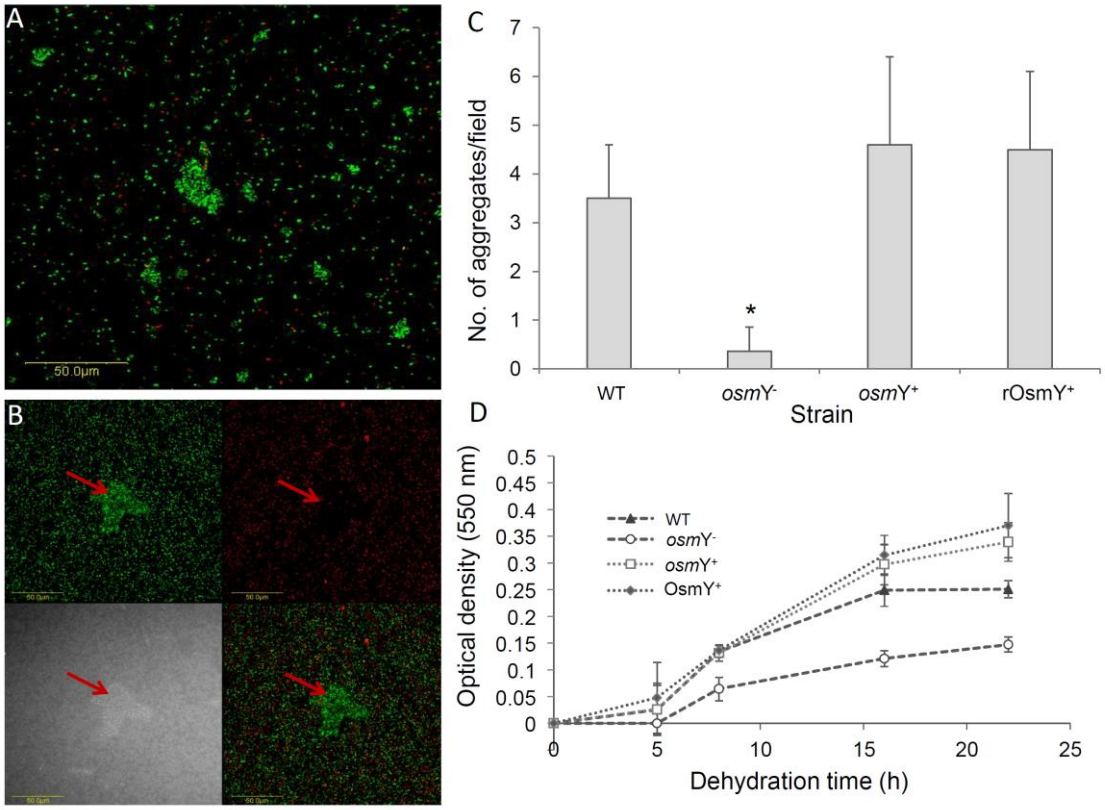

Fig. 6. Biofilm formation during dehydration. (A) Dehydrated STm cells were stained after 22h with the LIVE/DEAD® *Bac*Light™ kit and visualized by confocal microscopy (35). Images showing live (green) and dead (red) cells. **(B)** Cell population after 4 weeks of cold-storage. Live (green) bacteria (top left), dead (red) bacteria (top right), overlay of the live and dead images (lower right) and a Nomarski image of the same field (lower left). Magnification x1600. Arrows point on bacterial aggregates. **(C)** Number of aggregates per microscopic field (x400) formed after t=22 h by the various strains. *osmY*$^-$, *osmY*$^+$ and OsmY$^+$ denote the *osmY* mutant strain, *osmY*$^-$ strain complemented with the recombinant rOsmY plasmid, and *osmY*$^-$ strain dehydrated in the presence of rOsmY (10 µg/ml), respectively. Data for each strain was obtained from a total of 300 microscopic fields and represent the mean of three independent experiments, each performed in duplicate. Error bars denote the standard deviation of the mean. The bar marked with asterisk indicates significant difference (One-way ANOVA, $P < 0.05$) in the number of aggregates from the WT. **(D)** Microtiter biofilm assay. STm cells suspended in 50 µl SDDW at 10^8 CFU/well were dehydrated and the mass of surface-associated bacteria was determined by the crystal violet assay at 5, 8, 16 and 22 h. The amount of surface-associated bacteria is presented as an optical density at 550 nm.

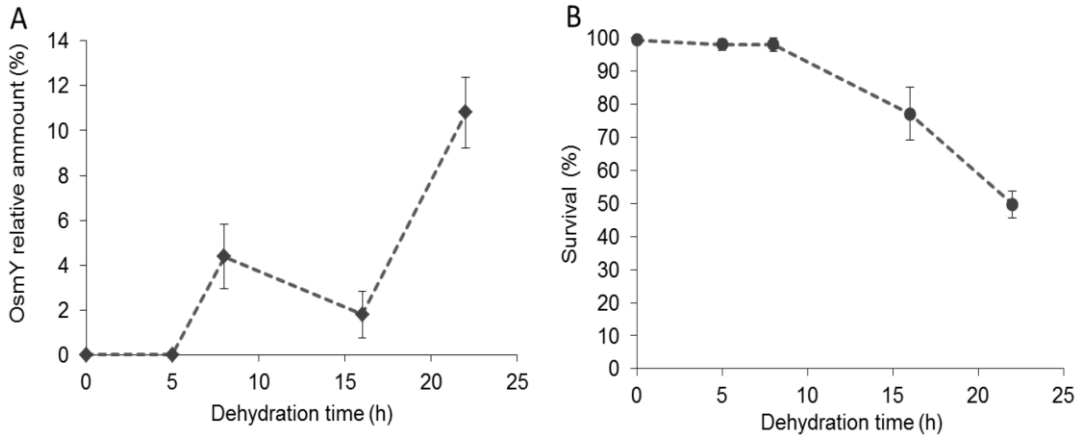

Fig. 7. Morphology of dehydrated bacteria. STm cells were dehydrated on polyethylene plastic discs, processed and visualized using scanning electron microscopy (SEM). Representative images are presented. **(A).** Morphology of WT, *osmY*⁻ mutant and *osmY*⁺ strain (*osmY*⁻ mutant complemented with the WT *osmY* gene). WT and *osmY*⁺ display clusters of cells interconnected via fimbriae. Arrows denote regions where cells are embedded in an EPS-like matrix. **(B)** High magnification images of individual dehydrated cells demonstrating a capsule-like structure on the surface of the WT and the *osmY*⁺ strain, but not on the *osmY*⁻ mutant strain. The WT and the *osmY*⁺ strains appear wider than the *osmY*⁻ mutant strain and are intimately attached to the surface via an amorphous substance. **(C)** *osmY*⁻ mutant cells were dehydrated in the presence of rOsmY (10 μg/ml) and visualized by SEM. A representative cell embedded within an EPS-like material is shown. Magnification is x20000, x80000 and x150000 for A, B and C, respectively.